MODULE II
CHROMATOGRAPHY

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for more advanced use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive.

- **Chromatography is a laboratory technique for the separation of a mixture.**

- **The basic principle is that components in a mixture have different tendencies to adsorb onto a surface or dissolve in a solvent.**

- **All chromatographic methods require one static part (the stationary phase) and one moving part (the mobile phase).**

- **The techniques rely on one of the following phenomena: adsorption; partition; ion exchange; or molecular exclusion**

- **Separation of the components, or solutes, of a mixture is on the basis of the relative amounts of each solute distributed between a moving fluid stream, called the mobile phase, and a contiguous stationary phase.**

- **The mobile phase may be either a liquid or a gas, while the stationary phase is either a solid or a liquid.**

- **The mixture is dissolved in a fluid called the *mobile phase,* which carries it through a structure holding another material called the *stationary phase.***

- **The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases.**

- **It is a separation process involving two phases, one stationary and the other mobile.**

- **Typically, the stationary phase is a porous solid (e.g., glass, silica, or alumina) that is packed into a glass or metal tube or that constitutes the walls of an open-tube capillary.**

- **The mobile phase flows through the packed bed or column.**

- **The sample to be separated is injected at the beginning of the column and is transported through the system by the mobile phase.**

- **In their travel through the column, the different substances distribute themselves according to their relative affinity for the two phases.**

- **The rate of travel is dependent on the values of the distribution coefficients, the components interacting more strongly with the stationary phase requiring longer time periods for elution (complete removal from the column).**

- **Thus, separation is based on differences in distribution behaviour reflected in different migration times through the column.**

- **The various chromatographic methods are characterized in terms of the mobile phase—gas: gas chromatography (GC); liquid: liquid chromatography (LC); supercritical fluid: supercritical-fluid chromatography (SFC). The methods are then further subdivided in terms of the stationary phase; thus, if the stationary phase is a solid adsorbent, there are methods such as gas-solid chromatography (GSC) and liquid-solid chromatography (LSC).**

- **A chromatograph is equipment that enables a sophisticated separation, e.g. gas chromatographic or liquid chromatographic separation.**
- **Chromatography is a physical method of separation that distributes components to separate between two phases, one stationary (stationary phase), the other (the mobile phase) moving in a definite direction.**
- **The eluate is the mobile phase leaving the column.**
- **The eluent is the solvent that carries the analyte.**
- **An eluotropic series is a list of solvents ranked according to their eluting power.**

•An **immobilized phase** is a stationary phase that is immobilized on the support particles, or on the inner wall of the column tubing.
•The **mobile phase** is the phase that moves in a definite direction. It may be a liquid (LC and Capillary Electrochromatography (CEC)), a gas (GC), or a supercritical fluid (supercritical-fluid chromatography, SFC). The mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column. In the case of HPLC the mobile phase consists of a non-polar solvent(s) such as hexane in normal phase or a polar solvent such as methanol in reverse phase chromatography and the sample being separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.

- The **solute** refers to the sample components in partition chromatography.
- The **solvent** refers to any substance capable of solubilizing another substance, and especially the liquid mobile phase in liquid chromatography.
- The **stationary phase** is the substance fixed in place for the chromatography procedure. Examples include the silica layer in thin layer chromatography
- The **detector** refers to the instrument used for qualitative and quantitative detection of analytes after separation

Preparative chromatography is used to purify sufficient quantities of a substance for further use, rather than analysis.

The **retention time** is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions. See also: Kovats' retention index

The **sample** is the matter analyzed in chromatography. It may consist of a single component or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the sample whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as waste.

Chromatography is based on the concept of partition coefficient. Any solute partitions between two immiscible solvents. When we make one solvent immobile (by adsorption on a solid support matrix) and another mobile it results in most common applications of chromatography. If the matrix support, or stationary phase, is polar (e.g. paper, silica etc.) it is forward phase chromatography, and if it is non-polar (C-18) it is reverse phase.

PAPER CHROMATOGRAPHY

- Paper chromatography uses paper as the stationary phase.
- The sample mixture is applied to a piece of filter paper, the edge of the paper is immersed in a solvent, and the solvent moves up the paper by capillary action.
- Components of the mixture are carried along with the solvent up the paper to varying degrees, depending on the compound's preference to be adsorbed onto the paper versus being carried along with the solvent.

- The paper is composed of cellulose to which polar water molecules are adsorbed, while the solvent is less polar, usually consisting of a mixture of water and an organic liquid.

- The paper is called the stationary phase while the solvent is referred to as the mobile phase.

- Performing a chromatographic experiment is basically a three-step process:
- 1) application of the sample,
- 2) "developing" the chromatogram by allowing the mobile phase to move up the paper,
- 3) calculating Rf values and making conclusions.

- **A drop of a solution of a mixture of dyes or inks is placed on a piece of chromatography paper and allowed to dry.**

- **The mixture separates as the solvent front advances past the mixture.**

- **Filter paper and blotting paper are frequently substituted for chromatography paper, but precision will be less.**

- **Separation is most efficient if the atmosphere is saturated in the solvent vapour.**

- **Paper chromatography works by the partition of solutes between water in the paper fibres (stationary phase) and the solvent (mobile phase).**

- **Common solvents that are used include pentane, propanone and ethanol.**

- **Mixtures of solvents are also used, including aqueous solutions, and solvent systems with a range of polarities can be made.**

- **The paper is removed when the solvent front approaches the top of the paper.**

•As each solute distributes itself (equilibrates) between the stationary and the mobile phase, the distance a solute moves is always the same fraction of the distance moved by the solvent.

•This fraction is variously called the retardation factor or the retention ratio, and is given the symbol R or Rf.

- For a given system at a known temperature, it is a characteristic of the component and can be used to identify components. An Rf value is a number that is defined as:

$$Rf = \frac{\text{distance traveled by component from application point}}{\text{distance traveled by solvent from application point}}$$

-

- **A particular compound will travel the same distance along the stationary phase by a specific solvent (or solvent mixture) given that other experimental conditions are kept constant –**

- **Temperature**

- **Chromatography medium, i.e same type and grade of Chromatography Paper**

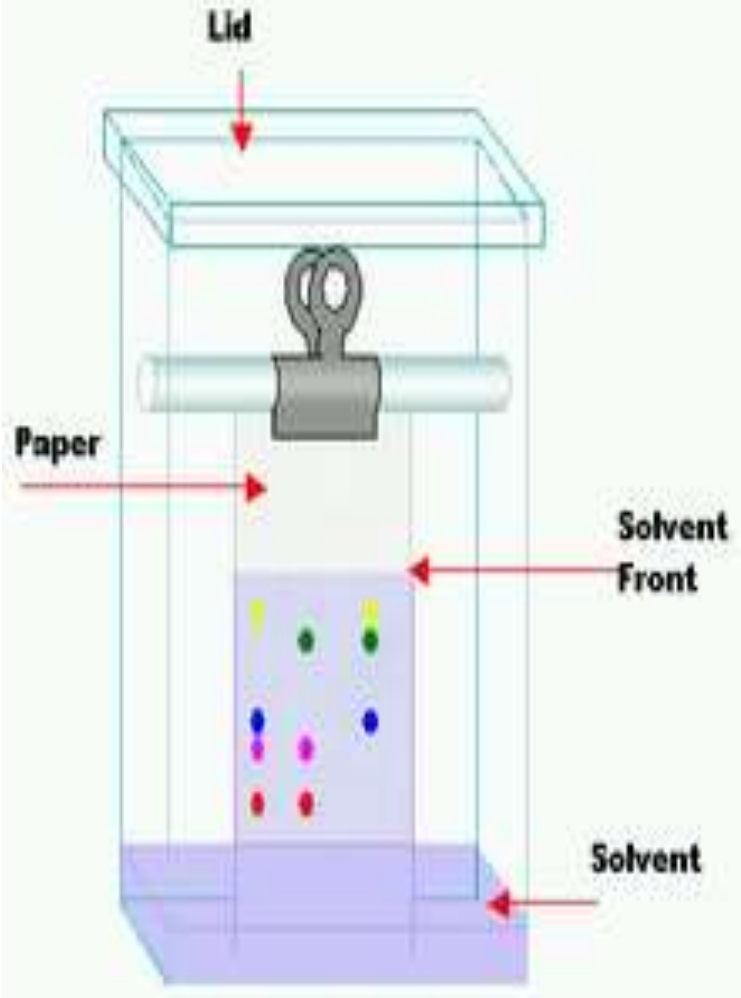
- **Solvent concentration and purity**

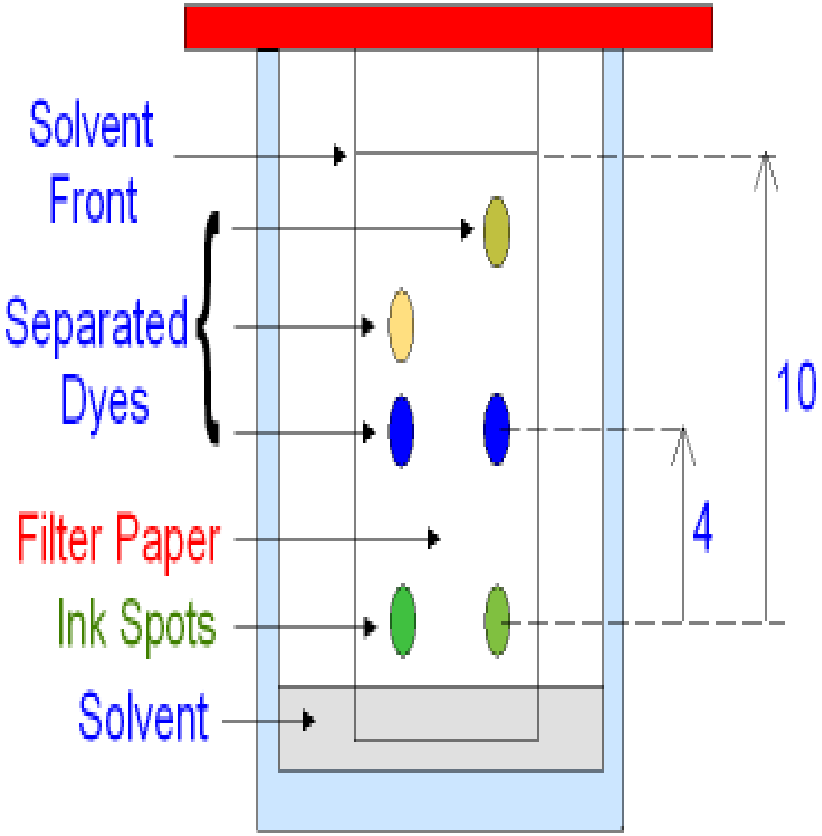
- **Amount of sample spotted on Chromatography medium**

• Every compound (dye, pigment, organic substance etc) have a specific Rf value for every specific solvent and solvent concentration.

•Rf values come very handy for identification because one can compare Rf values of the unknown sample (or its constituents) with Rf Values of known compounds. So as long as the correct solvent and type of chromatography paper are used, a component can be identified from its retention ratio

•The distance traveled by the spot is measured to the middle of the spot.


Lid
Paper
Solvent
Front
Solvent


Solvent
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Separated
Dyes
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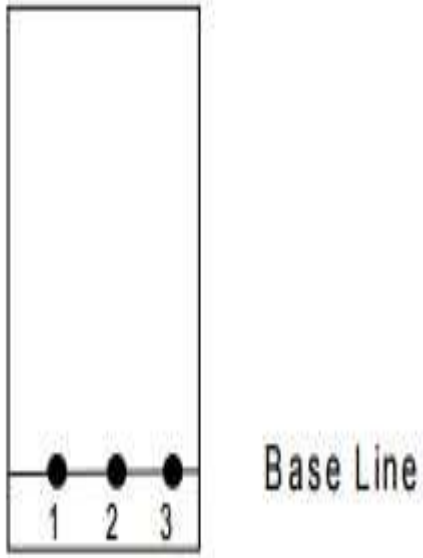
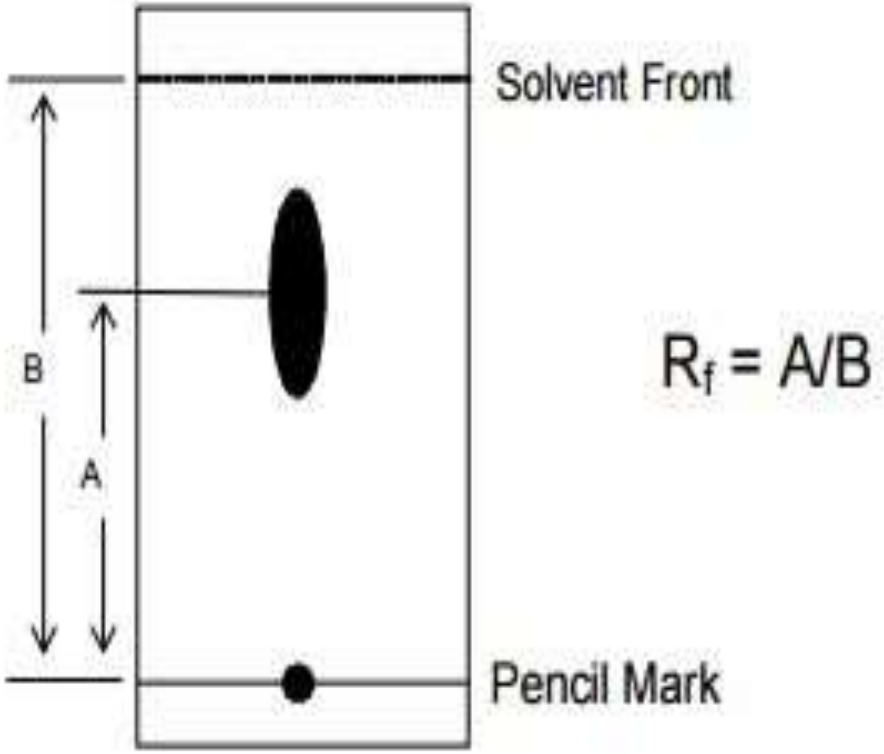
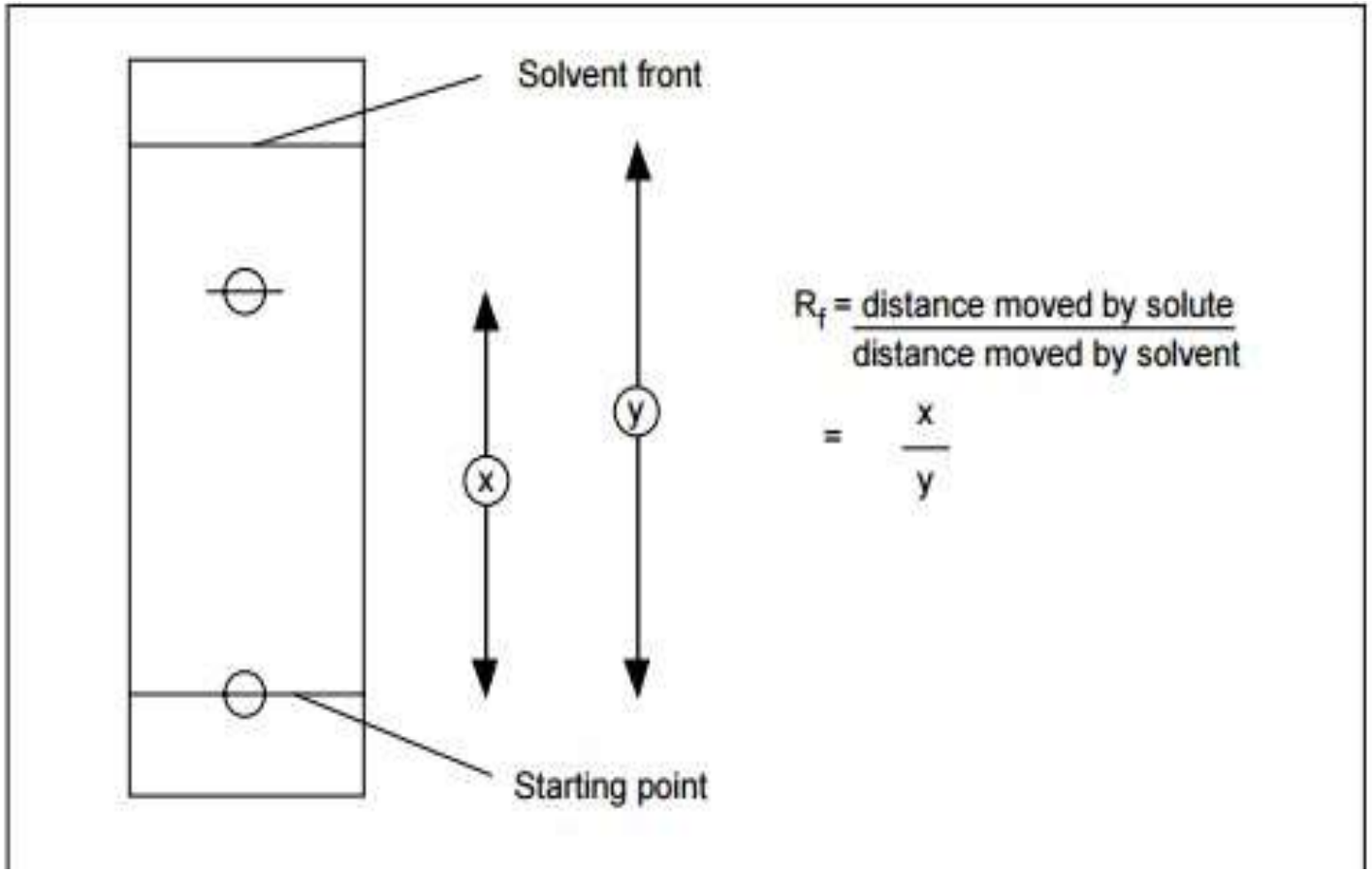
10
4





$R_f = A/B$


Solvent front
x
y
Starting point
R_f = distance moved by solute / distance moved by solvent
= x / y

- **It is possible that two solutes have the same Rf values using one solvent, but different values using another solvent (eg this occurs with some amino acids).**

- **This means that if a multi component system is not efficiently separated by one solvent the chromatogram can be dried, turned through 90^{o}, and run again using a second solvent.**

THIN LAYER CHROMATOGRAPHY (TLC)

- **Thin layer chromatography is similar to paper chromatography, but the stationary phase is a thin layer of a solid such as alumina or silica supported on an inert base such as glass, aluminum foil or insoluble plastic.**

- **The mixture is 'spotted' at the bottom of the TLC plate and allowed to dry.**

- **The plate is placed in a closed vessel containing solvent (the mobile phase) so that the liquid level is below the spot.**

- **TLC has advantages over paper chromatography in that its results are more reproducible, and that separations are very efficient because of the much smaller particle size of the stationary phase.**

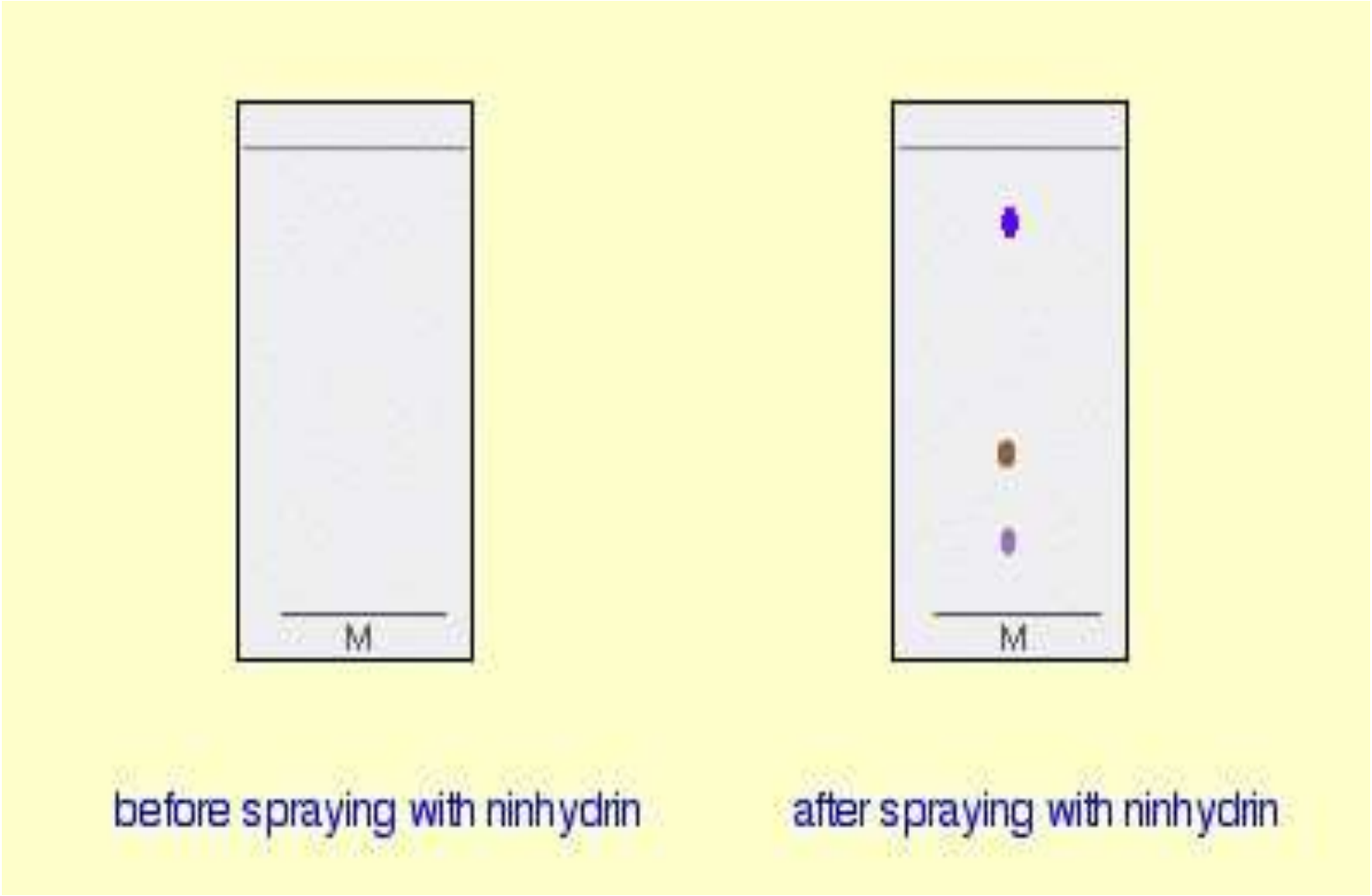
- **The solvent ascends the plate by capillary action, the liquid filling the spaces between the solid particles.**

- **This technique is usually done in a closed vessel to ensure that the atmosphere is saturated with solvent vapour and that evaporation from the plate is minimised before the run is complete.**

- **The plate is removed when the solvent front approaches the top of the plate and the position of the solvent front recorded before it is dried (this allows the Rf value to be calculated).**

- **TLC has applications in industry in determining the progress of a reaction by studying the components present; and in separating reaction intermediates.**

- **Many spots are not visible without the plates being 'developed'. This usually involves spraying with a solution that is reversibly adsorbed or reacts in some way with the solutes e.g, ninhydrin is used for identification of amino acids.**


M
M
before spraying with ninhydrin
after spraying with ninhydrin

The stationary phase – silica gel

- Silica gel is a form of silicon dioxide (silica).

- The silicon atoms are joined via oxygen atoms in a giant covalent structure. However, at the surface of the silica gel, the silicon atoms are attached to -OH groups.

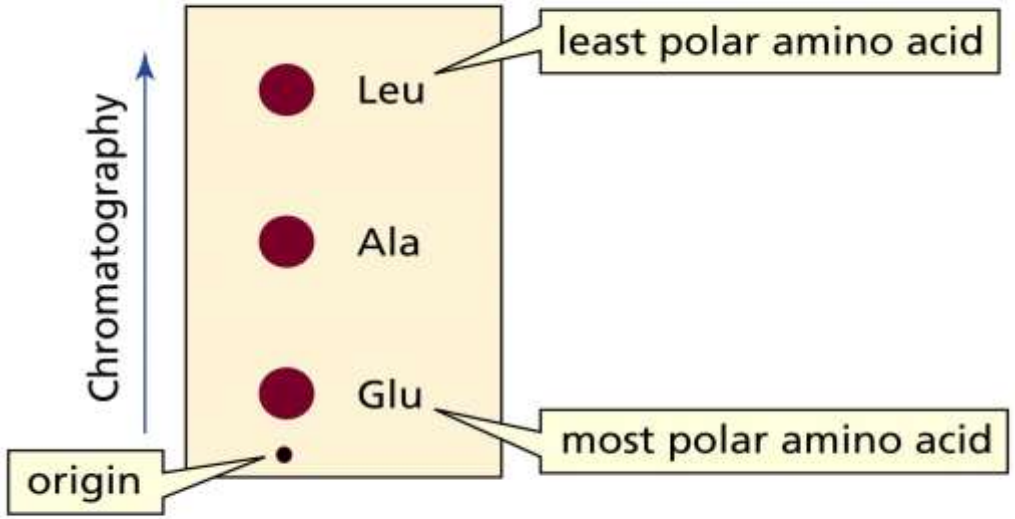
- The surface of the silica gel is very polar and, because of the -OH groups, can form hydrogen bonds with suitable compounds around it as well as van der Waals dispersion forces and dipole-dipole attractions.

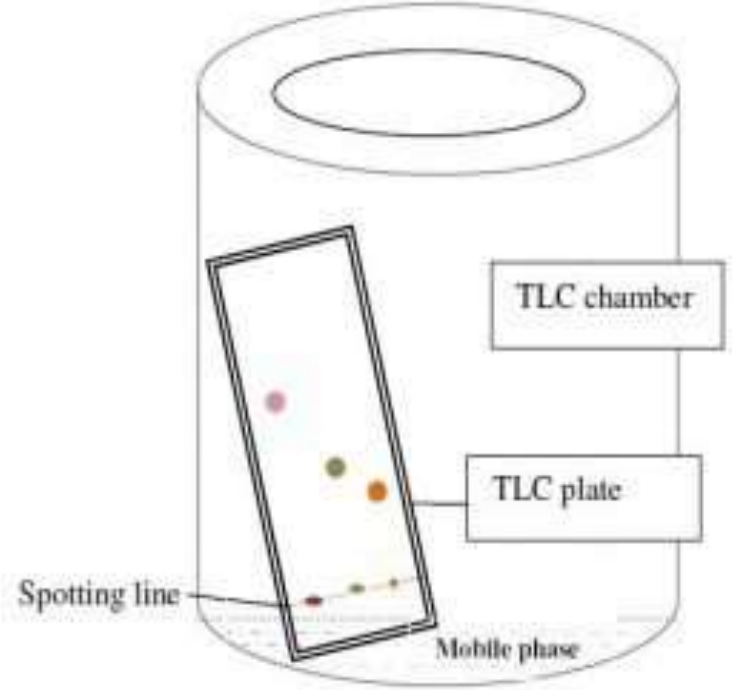
- **Adsorption is the name given to one substance forming some sort of bonds to the surface of another one.**

- **The compound can only travel up the plate during the time that it is dissolved in the solvent.**

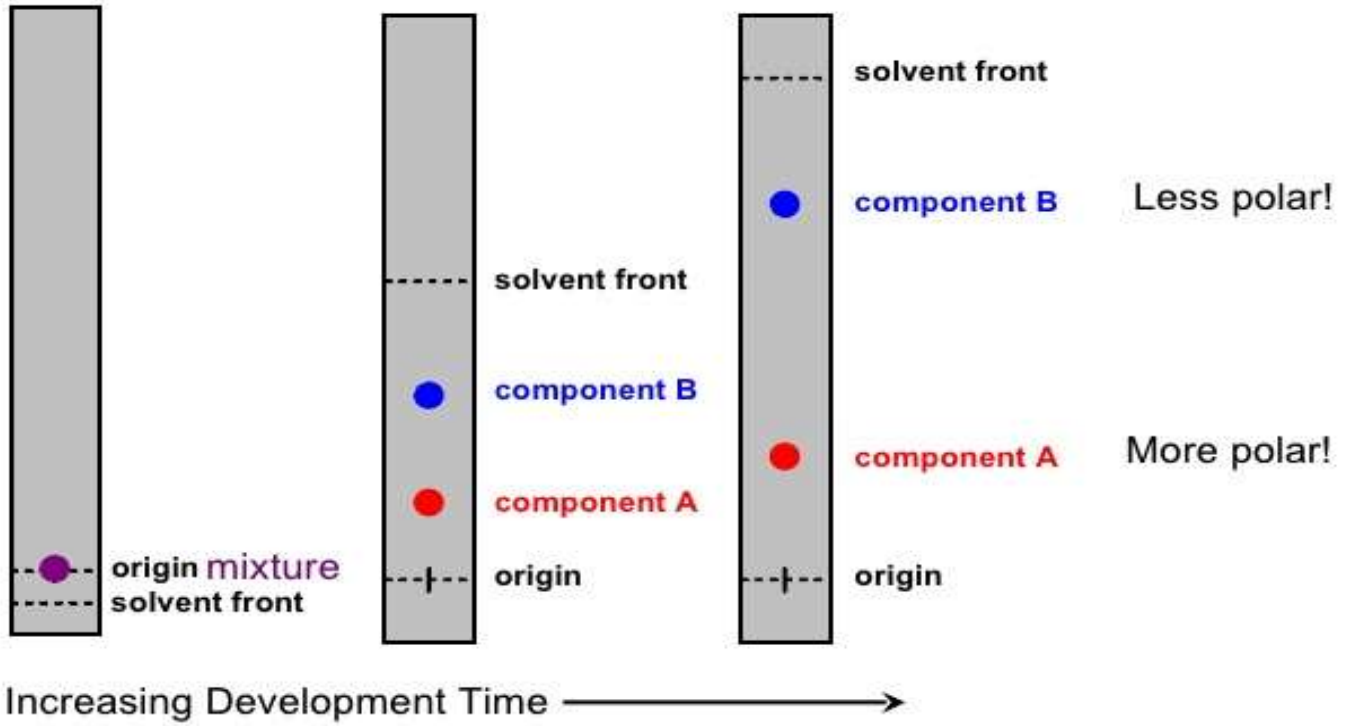
- **While it is adsorbed on the silica gel, it is temporarily stopped - the solvent is moving on without it. That means that the more strongly a compound is adsorbed, the less distance it can travel up the plate. i.e., the compound which can hydrogen bond will adsorb more strongly than the one dependent on van der Waals interactions, and so won't travel so far up the plate.**

•The other commonly used stationary phase is alumina - aluminium oxide. The aluminium atoms on the surface of this also have -OH groups attached. Anything we say about silica gel therefore applies equally to alumina.





Thin-Layer Chromatography: A Two-Component Mixture



ION EXCHANGE CHROMATOGRAPHY

- Ion exchange chromatography is used to remove ions of one type from a mixture and replace them by ions of another type.

- Process that separates ions and polar molecules based on their affinity to the ion exchanger.

- It works on almost any kind of charged molecule—including large proteins, small nucleotides, and amino acids.

- It is often used in protein purification, water analysis etc.

•Ion-exchange chromatography which is designed specifically for the separation of differently charged or ionizable compounds comprises from mobile and stationary phases similar to other forms of column based liquid chromatography techniques.

•Mobile phases consist an aqueous buffer system into which the mixture to be resolved. The stationary phase usually made from inert organic matrix chemically derivative with ionizable functional groups (fixed ions) which carry displaceable oppositely charged ion.

- **The water-soluble and charged molecules such as proteins, amino acids, and peptides bind to moieties which are oppositely charged by forming ionic bonds to the insoluble stationary phase.**

- **The column is packed with porous beads of a resin that will exchange either cations or anions.**

- **There is one type of ion on the surface of the resin and these are released when other ions are bound in their place – eg a basic anion exchange resin might remove nitrate ions (NO_3^-) from a solution and replace them with hydroxide ions (OH^-).**

- **A cationic stationary phase is used to separate anions and an anionic stationary phase is used to separate cations.**

- **Cation exchange chromatography is used when the desired molecules to separate are cations and anion exchange chromatography is used to separate anions.**

- **Many of the resins used are based on phenylethene (styrene) polymers with cross linking via 1,4-bis-ethenylbenzene.**

- **The reasons for the success of ion exchange are its widespread applicability, its high resolving power, its high capacity, and the simplicity and controllability of the method.**

- **If a protein has a net positive charge at pH 7, it will usually bind to a column of beads containing carboxylate groups, whereas a negatively charged protein will not.**

- **The bound protein can then be eluted (released) by increasing the concentration of sodium chloride or another salt in the eluting buffer; sodium ions compete with positively charged groups on the protein for binding to the column.**

- **Proteins that have a low density of net positive charge will tend to emerge first, followed by those having a higher charge density.**

- **This procedure is also referred to as *cation exchange* to indicate that positively charged groups will bind to the anionic beads.**

- **Positively charged proteins (cationic proteins) can be separated by chromatography on negatively charged carboxymethylcellulose (CM-cellulose) columns.**

- **Conversely, negatively charged proteins (anionic proteins) can be separated by *anion exchange* on positively charged diethylaminoethylcellulose (DEAE-cellulose) columns.**

- During anion-exchange chromatography for example, negatively charged protein analytes can be competitively displaced by the addition of negatively charged chloride ions (e.g. from sodium chloride).

- By gradually increasing the salt concentration in the mobile phase, the affinity of interaction between the salt ions and the functional groups will eventually exceed that which exists between the protein charges and the functional groups, resulting in protein displacement and elution.

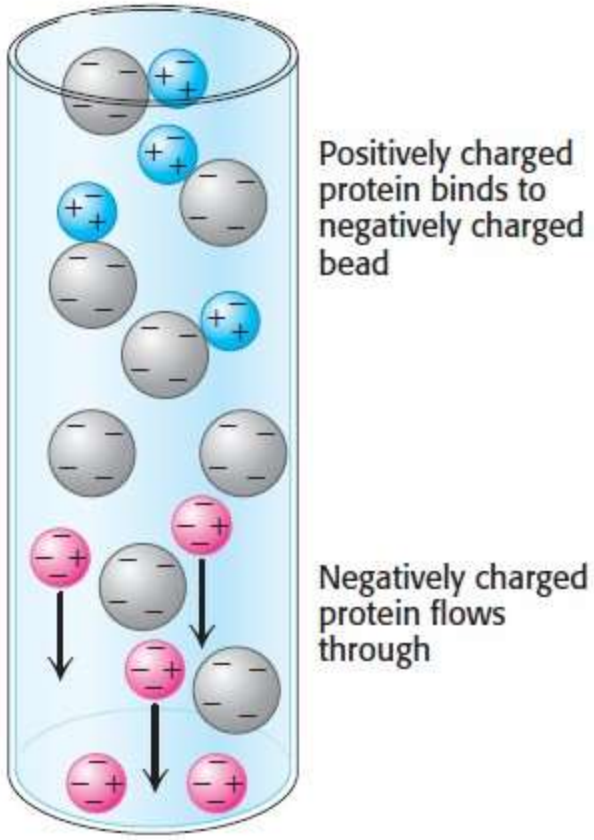
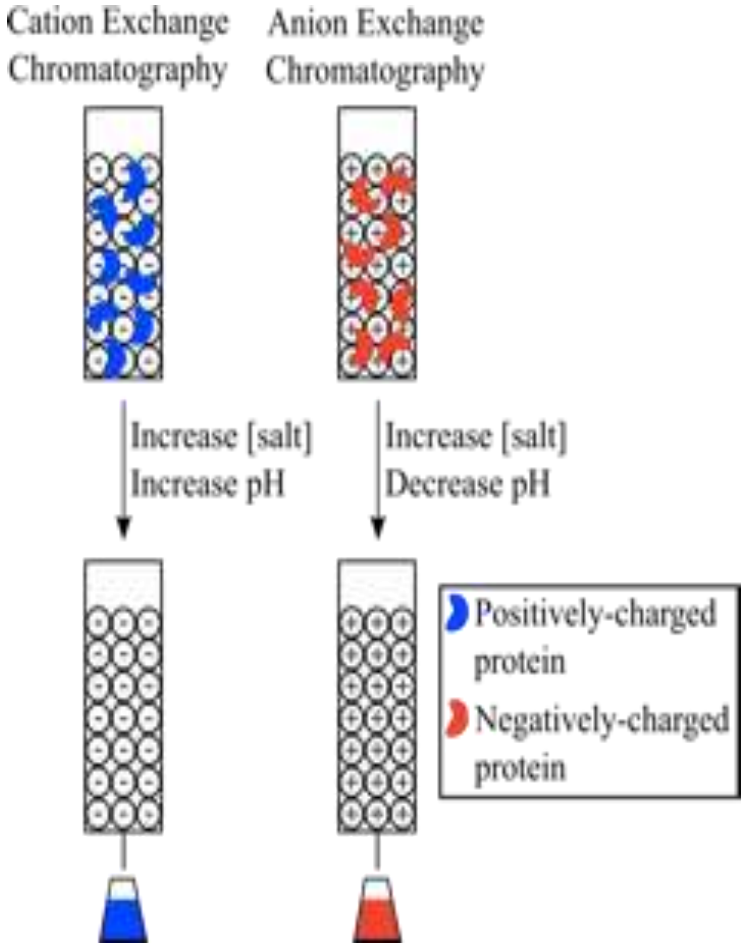


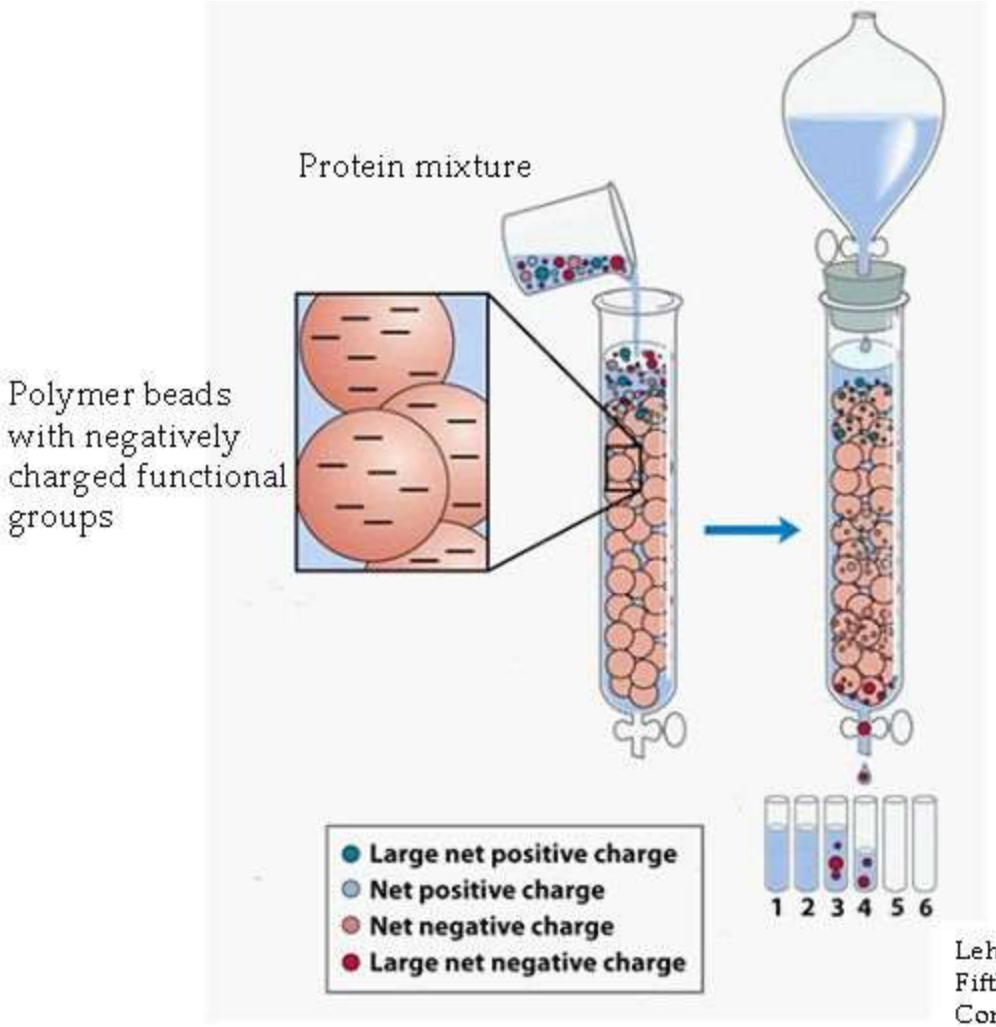
Figure 3.4 Ion-exchange chromatography. This technique separates proteins mainly according to their net charge.

•Separation in ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge.

•Bound protein analytes can be eluted (desorption) in one of two possible ways: (i) pH; (ii) ionic strength.

•Changing the mobile phase pH alters the net charge of the bound protein, and thus its matrix binding capacity. More commonly, increasing the concentration of a similarly charged species within the mobile phase can compete with and ultimately displace the bound ionic species.





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GEL PERMEATION CHROMATOGRAPHY (GPC)

- Gel permeation chromatography (GPC) is a type of size exclusion chromatography (SEC), that separates analytes on the basis of size.
- The technique is often used for the analysis of polymers.
- The GPC column is packed with porous beads of controlled porosity and particle size
- Polymer is prepared as a dilute solution in the eluent and injected into the system

- **This differs from other separation techniques which depend upon chemical or physical interactions to separate analytes.**
- **Separation occurs via the use of porous beads packed in a column.**
- **The smaller analytes can enter the pores more easily and therefore spend more time in these pores, increasing their retention time.**
- **These smaller molecules spend more time in the column and therefore will elute last.**

- **Conversely, larger analytes spend little if any time in the pores and are eluted quickly.**

- **All columns have a range of molecular weights that can be separated.**

- **If an analyte is either too large or too small, it will be either not retained or completely retained, respectively.**

- **Analytes that are not retained are eluted with the free volume outside of the particles, while analytes that are completely retained are eluted with volume of solvent held in the pores.**

- **Large molecules are not able to permeate all of the pores and have a shorter residence time in the column.**

- **Small molecules permeate deep into the porous matrix and have a long residence time in the column.**

- **Polymer molecules are separated according to molecular size, eluting largest first, smallest last.**

- **The separation is purely a physical partitioning, there is no interaction or binding.**
- **The calibration curve describes how different size molecules elute from the column.**

Instrumentation

- Gel permeation chromatography is conducted almost exclusively in chromatography columns.

- Samples are dissolved in an appropriate solvent, in the case of GPC these tend to be organic solvents and after filtering the solution it is injected onto a column.

- The separation of multi-component mixture takes place in the column.

- **The constant supply of fresh eluent to the column is accomplished by the use of a pump.**

- **Since most analytes are not visible to the naked eye a detector is needed. Often multiple detectors are used to gain additional information about the polymer sample.**

- **The availability of a detector makes the fractionation convenient and accurate.**

- **Gels** are used as stationary phase for GPC. The pore size of a gel must be carefully controlled in order to be able to apply the gel to a given separation.

- Other desirable properties of the gel forming agent are the absence of ionizing groups and, in a given solvent, low affinity for the substances to be separated.

- Commercial gels like PLgel, Sephadex, Bio-Gel (cross-linked polyacrylamide), agarose gel and Styragel are often used based on different separation requirements

- **The column used for GPC is filled with a microporous packing material. The column is filled with the gel.**

- **The eluent (mobile phase) should be a good solvent for the polymer, should permit high detector response from the polymer and should wet the packing surface.**

- **The most common eluents in for polymers that dissolve at room temperature GPC are tetrahydrofuran (THF), *o*-dichlorobenzene and trichlorobenzene at 130–150 °C for crystalline polyalkynes and *m*-cresol and *o*-chlorophenol at 90 °C for crystalline condensation polymers such as polyamides and polyesters.**

- In GPC, the concentration by weight of polymer in the eluting solvent may be monitored continuously with a detector.

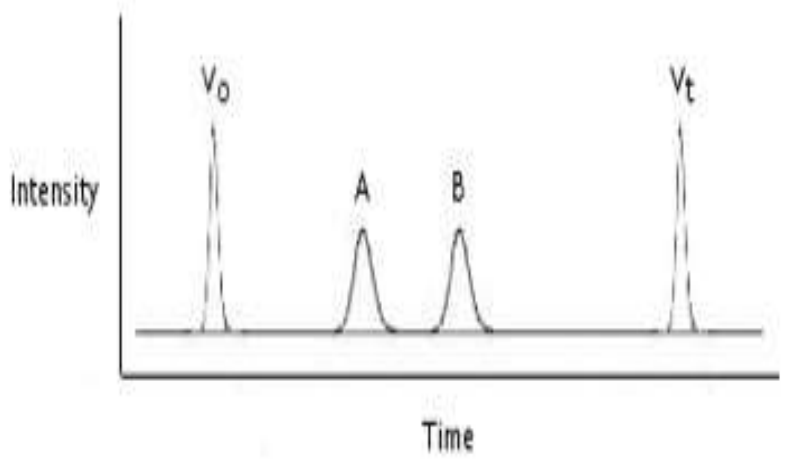
- There are many detector types available and they can be divided into two main categories.

- The first is concentration sensitive detectors which includes UV absorption, differential refractometer (DRI) or refractive index (RI) detectors, infrared (IR) absorption and density detectors.

- The second category is molecular weight sensitive detectors, which include low angle light scattering detectors (LALLS) and multi angle light scattering (MALLS).

- **The resulting chromatogram is therefore a weight distribution of the polymer as a function of retention volume.**

- **The most sensitive detector is the differential UV photometer and the most common detector is the differential refractometer (DRI).**



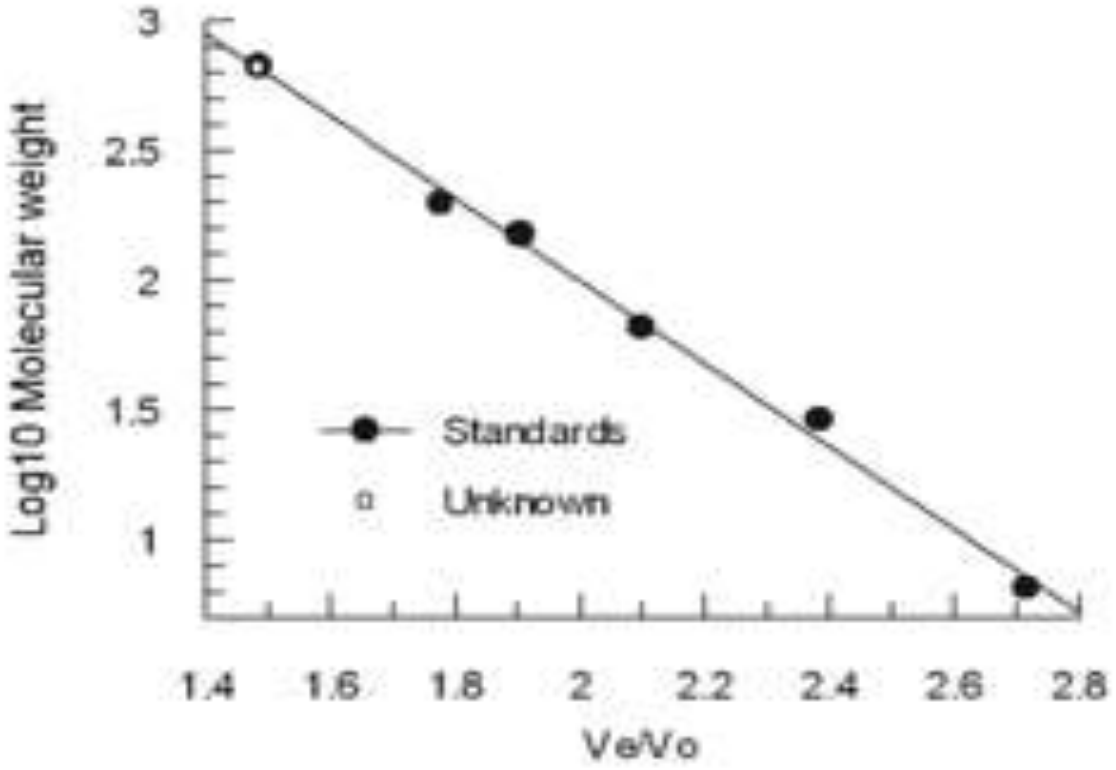
GPC Chromatogram; V_o= no retention, V_t= complete retention, A and B = partial retention

Data Analysis

- Gel permeation chromatography (GPC) has become the most widely used technique for analyzing polymer samples in order to determine their molecular weights and weight distributions.

- A calibration curve can be obtained by plotting the logarithm of the molecular weight versus the retention time or volume.

- Once the calibration curve is obtained, the gel permeation chromatogram of any other polymer can be obtained in the same solvent and the molecular weights (usually M_n and M_w) and the complete molecular weight distribution for the polymer can be determined.



Standardization of a size exclusion column.

Advantages of GPC

- it has a well-defined separation time due to the fact that there is a final elution volume for all unretained analytes.
- Since the analytes do not interact chemically or physically with the column, there is a lower chance for analyte loss to occur.
- GPC provides a more convenient method of determining the molecular weights of polymers.

Disadvantages of GPC

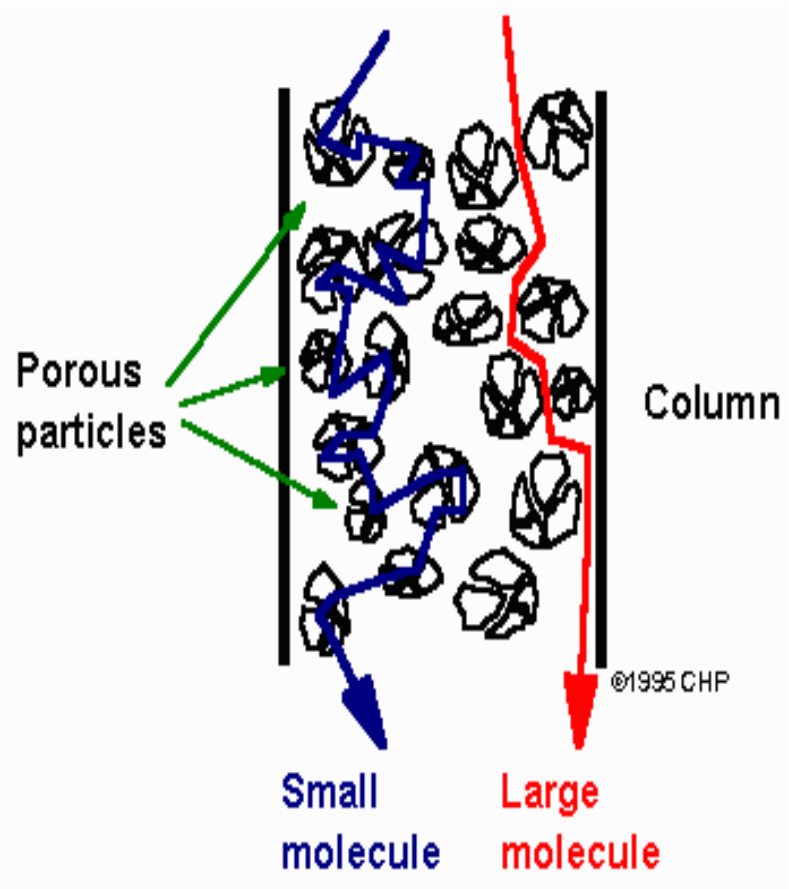
- There is a limited number of peaks that can be resolved within the short time scale of the GPC run.

- As a technique GPC requires around at least a 10% difference in molecular weight for a reasonable resolution of peaks to occur.

Application

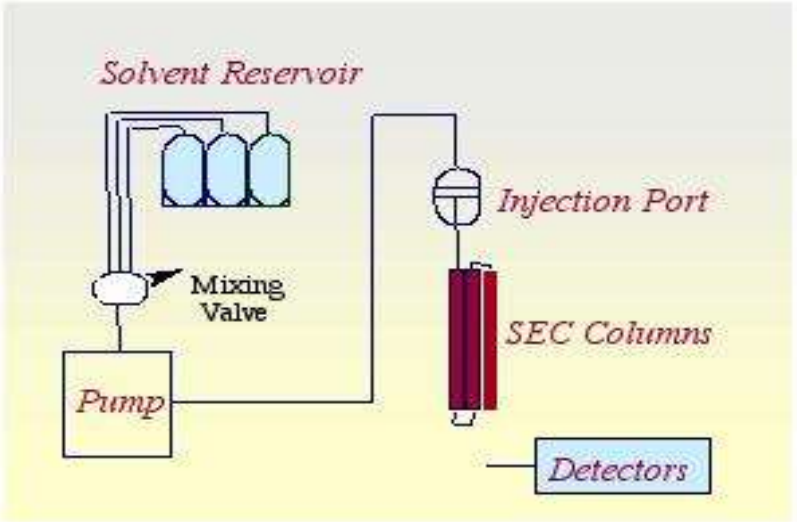
- **GPC is often used to determine the relative molecular weight of polymer samples as well as the distribution of molecular weights.**

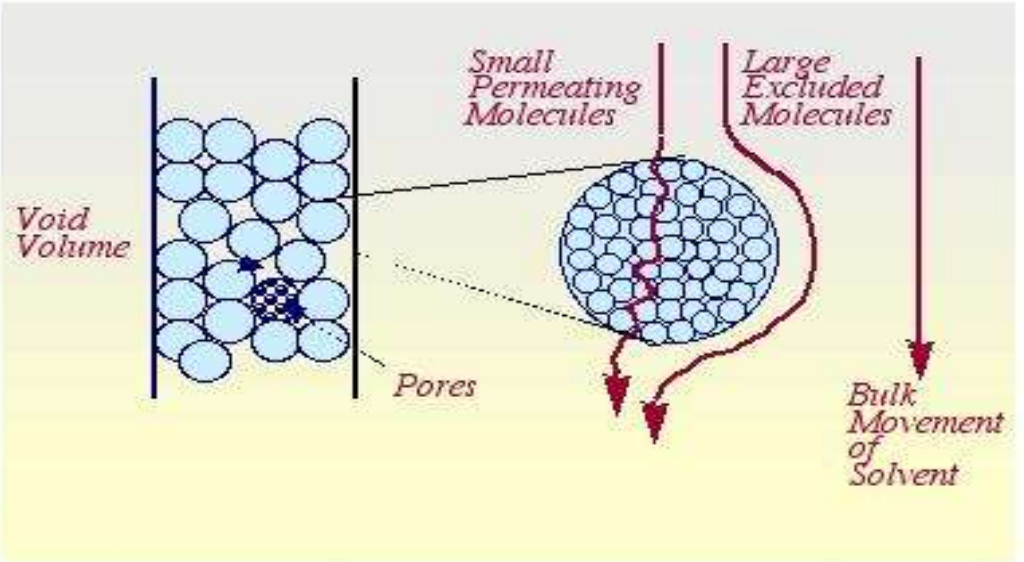
- **What GPC truly measures is the molecular volume and shape function as defined by the intrinsic viscosity.**



A typical GPC instrument including: A. Autosampler, B. Column, C. Pump, D. RI detector, E. UV-vis detector

Size Exclusion(or Gel Permeation) Chromatography





For a given volume of solvent flow, molecules of different size travel different path lengths within the column.The smaller ones travel greater distances than the larger molecules due to permeation into the molecular maze.Hence, the large molecules are eluted first from the column, followed by smaller and smaller molecules.

AFFINITY CHROMATOGRAPHY

- It is a method of separating biochemical mixtures based on a highly specific interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand.

- Separation of a desired protein using affinity chromatography relies on the reversible interactions between the protein to be purified and the affinity ligand coupled to chromatographic matrix.

- **Most of the proteins have an inherent recognition site that can be used to select the appropriate affinity ligand.**

- **The binding between the protein of interest and the chosen ligand must be both specific and reversible.**

- **It is based on the binding affinity of a protein. The beads in the column have a covalently attached chemical group. A protein with affinity for this particular chemical group will bind to the beads in the column, and its migration will be retarded as a result.**

Principle

- The stationary phase is typically a gel matrix, often of agarose; a linear sugar molecule derived from algae.
- Usually the starting point is an undefined heterogeneous group of molecules in solution, such as a cell lysate, growth medium or blood serum.
- The molecule of interest will have a well known and defined property, and can be exploited during the affinity purification process.

- The process itself can be thought of as an entrapment, with the target molecule becoming trapped on a solid or stationary phase or medium.

- The other molecules in the mobile phase will not become trapped as they do not possess this property.

- The stationary phase can then be removed from the mixture, washed and the target molecule released from the entrapment in a process known as elution.

- Possibly the most common use of affinity chromatography is for the purification of **recombinant proteins.**

- **Affinity purification involves 3 main steps:**

1. **Incubation of a crude sample with the affinity support to allow the target molecule in the sample to bind to the immobilized ligand.**

2. **Washing away non-bound sample components from the support.**

3. **Elution (dissociation and recovery) of the target molecule from the immobilized ligand by altering the buffer conditions so that the binding interaction no longer occurs.**

- For eg., the plant protein concanavalin A is a carbohydrate-binding protein, or lectin, that has affinity for glucose. When a crude extract is passed through a column of beads containing covalently attached glucose residues, concanavalin A binds to the beads, whereas most other proteins do not.

- The bound concanavalin A can then be released from the column by adding a concentrated solution of glucose. The glucose in solution displaces the column-attached glucose residues from binding sites on concanavalin A.

- **Affinity chromatography is a powerful means of isolating transcription factors—proteins that regulate gene expression by binding to specific DNA sequences.**

- **A protein mixture is passed through a column containing specific DNA sequences attached to a matrix; proteins with a high affinity for the sequence will bind and be retained. In this instance, the transcription factor is released by washing with a solution containing a high concentration of salt.**

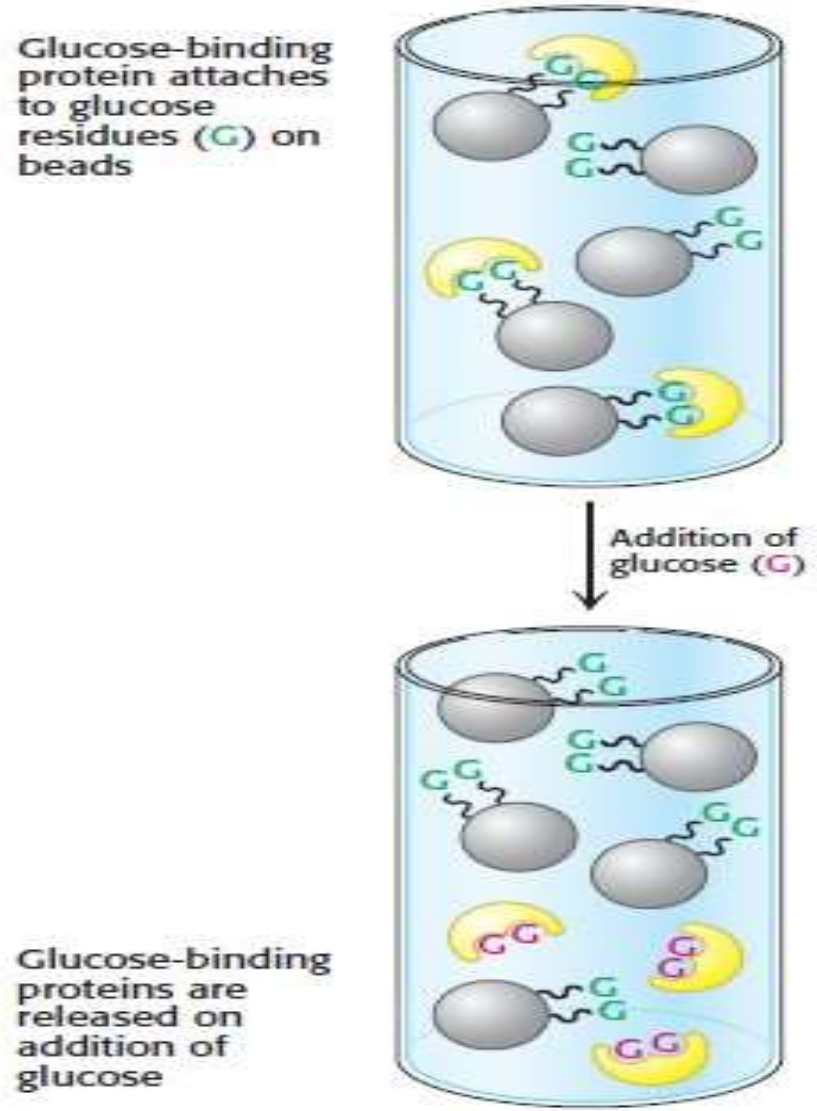


Figure 3.5 Affinity chromatography. Affinity chromatography of concanavalin A (shown in yellow) on a solid support containing covalently attached glucose residues (G).

Affinity supports (matrix)

- Traditionally, affinity chromatography support materials have consisted of porous support materials such as agarose, polymethacrylate, polyacrylamide, cellulose, and silica. Some supports may be available with common affinity ligands already immobilized (e.g. protein A, Cibacron Blue, heparin).

- Chemical inertness of the support material requires that the affinity support bind only the molecule of interest and have little or no nonspecific binding.

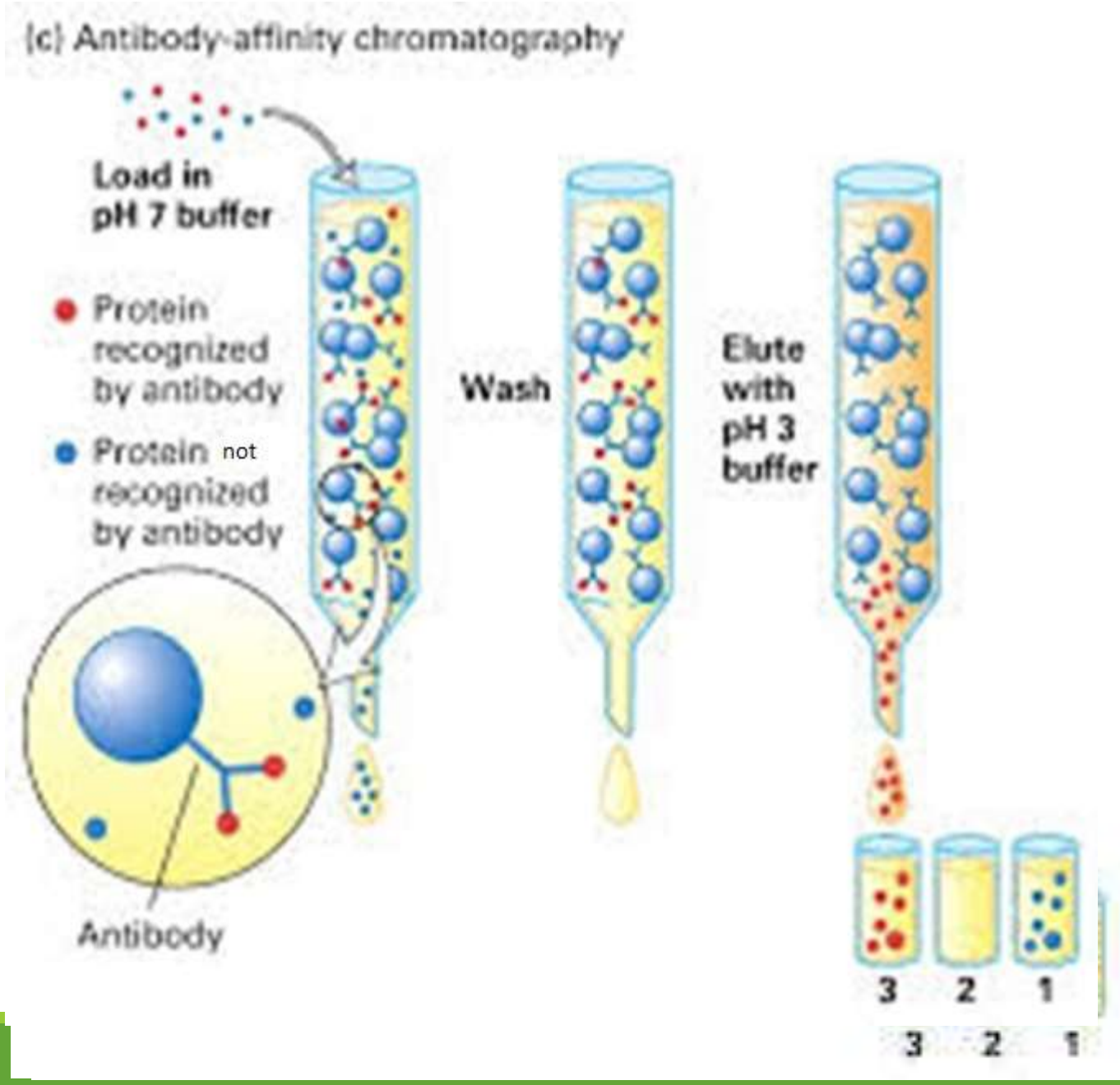
- **A support material must be chemically stable under normal operating conditions.**

- **Support materials must be able to withstand the backpressures encountered during normal separations without compressing.**

- **Particle size is also considered when choosing a support material. Ideally, small particle sizes are desired to limit mass transfer effects and limit band broadening.**

Uses

- Purify and concentrate a substance from a mixture into a buffering solution
- Reduce the amount of a substance in a mixture
- Discern what biological compounds bind to a particular substance
- Purify and concentrate an enzyme solution.


(c) Antibody-affinity chromatography
Load in pH 7 buffer
Protein recognized by antibody
Protein not recognized by antibody
Antibody
Wash
Elute with pH 3 buffer
3
2
1
3
2
1

GAS CHROMATOGRAPHY

- **Gas chromatography (GC) is a common type of chromatography used in analytical chemistry for separating & analyzing compounds that can be vaporized without decomposition.**

- **Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture**

- **Specifically gas-liquid chromatography - involves a sample being vapourised and injected onto the head of the chromatographic column.**

- **The sample is transported through the column by the flow of inert, gaseous mobile phase.**

- **The column itself contains a liquid stationary phase which is adsorbed onto the surface of an inert solid.**

Instrumental components

1. **Carrier gas**

- **The carrier gas must be chemically inert. Commonly used gases include nitrogen, helium, argon, and carbon dioxide.**

- **The choice of carrier gas is often dependent upon the type of detector which is used.**

- **The carrier gas system also contains a molecular sieve to remove water and other impurities.**

- **Helium remains the most commonly used carrier gas in about 90% of instruments although hydrogen is preferred for improved separations.**

- **The *stationary phase* is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column.**

- **The instrument used to perform gas chromatography is called a *gas chromatograph* (or "aerograph", "gas separator").**

- **The separation of compounds is based on the different strengths of interaction of the compounds with the stationary phase.**

- **The stronger the interaction is, the longer the compound interacts with the stationary phase, and the more time it takes to migrate through the column.**

- **Low boiling solvents (i.e., diethyl ether, dichloromethane) are used as solvents to dissolve the sample.**

- for example, the compound X interacts stronger with the stationary phase, and therefore lacks behind compound O in its movement through the column. As a result, compound O has a much shorter retention time than compound X.

- The sample is introduced in the liquid/gas form with the help of GC syringe into the injection port, it gets vaporized at injection port then passes through column with the help of continuously flowing carrier stream (mobile phase), and gets separated/detected at the detection port with suitable temperature programming. We visualize this on computer in the form of peaks.

- Different chemical constituents of the sample travel through the column at different rates depending upon,

1. Physical properties
2. Chemical properties,
3. Interaction with a specific column filling (stationary phase).

- As the chemicals exit the end of the column, they are detected and identified electronically.

- The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (retention time).

Three types of capillary columns are commonly used in gas chromatography:

1. Wall Coated Open Tubular (WCOT)

- Internal wall of capillary is coated with a very fine film of liquid stationary phase.

2. Surface Coated Open Tubular (SCOT)

- Capillary tube wall is lined with a thin layer of solid support on to which liquid phase is adsorbed. The separation efficiency of SCOT columns is more than WCOT columns because of increased surface area of the stationary phase coating.

3. Fused Silica Open Tubular (FSOT)

- Walls of capillary fused silica tubes are strengthened by a polyimide coating. These are flexible and can be wound into coils.

Mobile phase

- Hydrogen is most efficient and providing the best separation. However, helium has a larger range of flow rates that are comparable to hydrogen in efficiency, with the added advantage that helium is non-flammable, and works with a greater number of detectors. Therefore, helium is the most common carrier gas used. Which gas to use is usually determined by the detector being used.

Sample injection

1. Direct injection with syringe - Both gaseous and liquid samples can be injected with a syringe.

- **In the simplest form the sample is first injected into a heated chamber where it is vaporized before it is transferred to the column.**

- **When packed columns are used, the first part of the column often serves as injection chamber, separately heated to an appropriate temperature.**

- For capillary columns a separate injection chamber is used from which only a small part of the vaporized/gaseous sample is transferred to the column, so called split-injection. This is necessary in order not to overload the column in regard to the sample volume.

2. On-column inlet; the sample is here introduced directly into the column in its entirety without heat, or at a temperature below the boiling point of the solvent.

- The low temperature condenses the sample into a narrow zone. The column and inlet can then be heated, releasing the sample into the gas phase.

- This ensures the lowest possible temperature for chromatography and keeps samples from decomposing above their boiling point.

3. **PTV injector** (Temperature-programmed sample introduction) - the technique as a method for the introduction of large sample volumes (up to 250 µL) in capillary GC.

- The temperature of the liner was chosen slightly below the boiling point of the solvent. The low-boiling solvent was continuously evaporated and vented through the split line.

4. **Gas source inlet or gas switching valve** - gaseous samples in collection bottles are connected to what is most commonly a six-port switching valve.

- The carrier gas flow is not interrupted while a sample can be expanded into a previously evacuated sample loop.

- Upon switching, the contents of the sample loop are inserted into the carrier gas stream.

5. P/T (Purge-and-Trap) system- An inert gas is bubbled through an aqueous sample causing insoluble volatile chemicals to be purged from the matrix.

- **The volatiles are 'trapped' on an absorbent column (known as a trap or concentrator) at ambient temperature.**

- **The trap is then heated and the volatiles are directed into the carrier gas stream.**

Detectors

- **The most common detectors are the Flame ionization detector (FID) and the thermal conductivity detector (TCD). While TCDs are essentially universal and can be used to detect any component other than the carrier gas.**

- **FIDs are sensitive primarily to hydrocarbons, and are more sensitive to them than TCD. However, a FID cannot detect water**

•Other detectors are sensitive only to specific types of substances, eg.,

1. Catalytic combustion detector (CCD), which measures combustible hydrocarbons and hydrogen.

2. Discharge ionization detector (DID), which uses a high-voltage electric discharge to produce ions.

3. Dry electrolytic conductivity detector (DELCD), which uses an air phase and high temperature to measure chlorinated compounds.

The Chromatogram

- As the components elute from the column they pass into a detector – where some physicochemical property of the analyte produces a response from the detector.

- This response is amplified and plotted against time – giving rise to a ‘chromatogram’

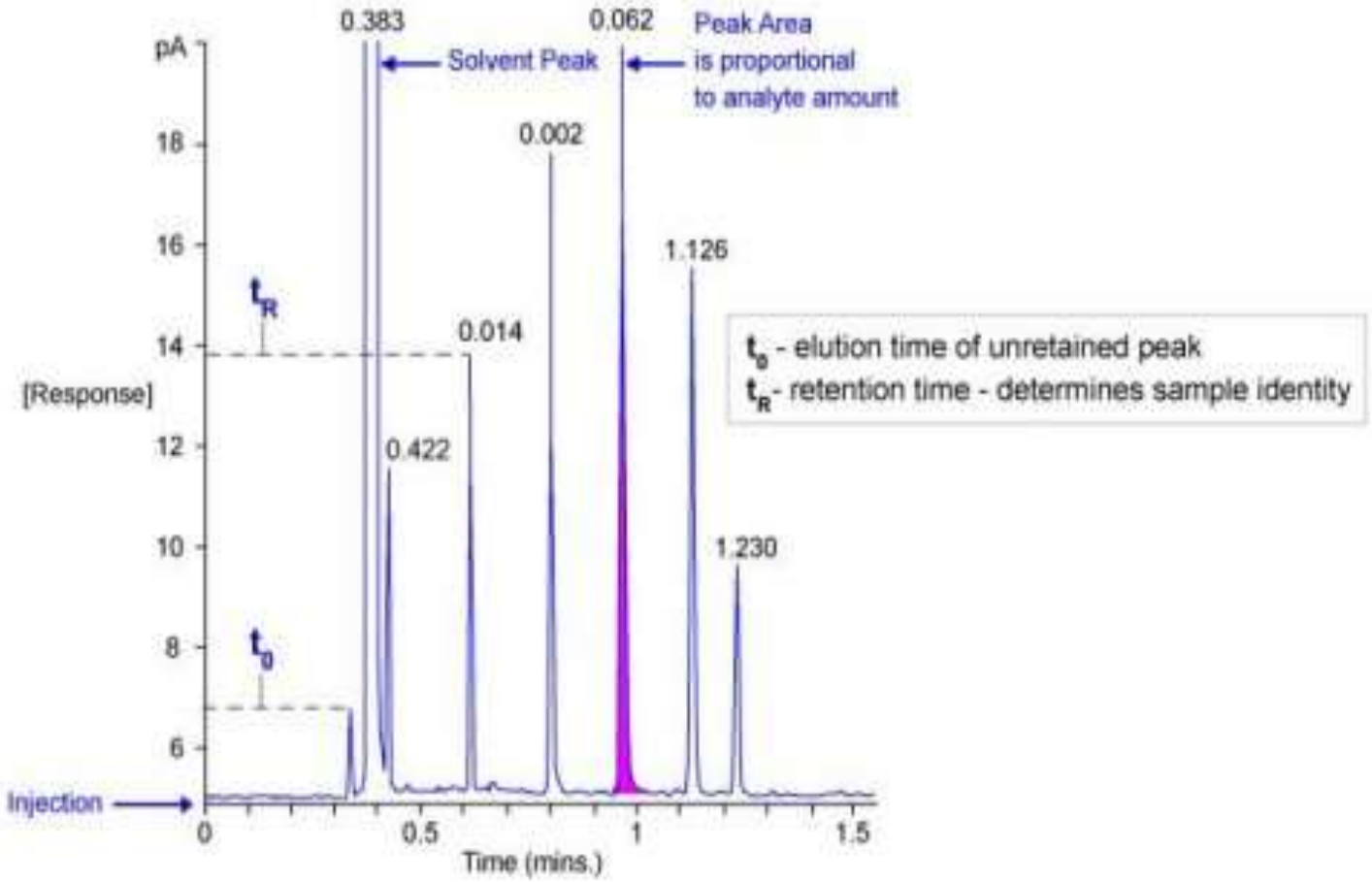


Figure 13: GC chromatogram.

- **Those compounds (analytes and sample components) that are retained elute as approximately 'Gaussian' shaped peaks later in the chromatogram.**

- **Retention times provide the qualitative aspect of the chromatogram and the retention time of a compound will always be the same under identical chromatographic conditions.**

- **The chromatographic peak height or peak area is related to the quantity of analyte. For determination of the actual amount of the compound, the area or height is compared against standards of known concentration.**

- **Most modern commercial GC systems operate in the following way**

 An inert carrier gas, such as helium, is supplied from gas cylinders to the GC where the pressure is regulated using manual or electronic (pneumatic) pressure controls

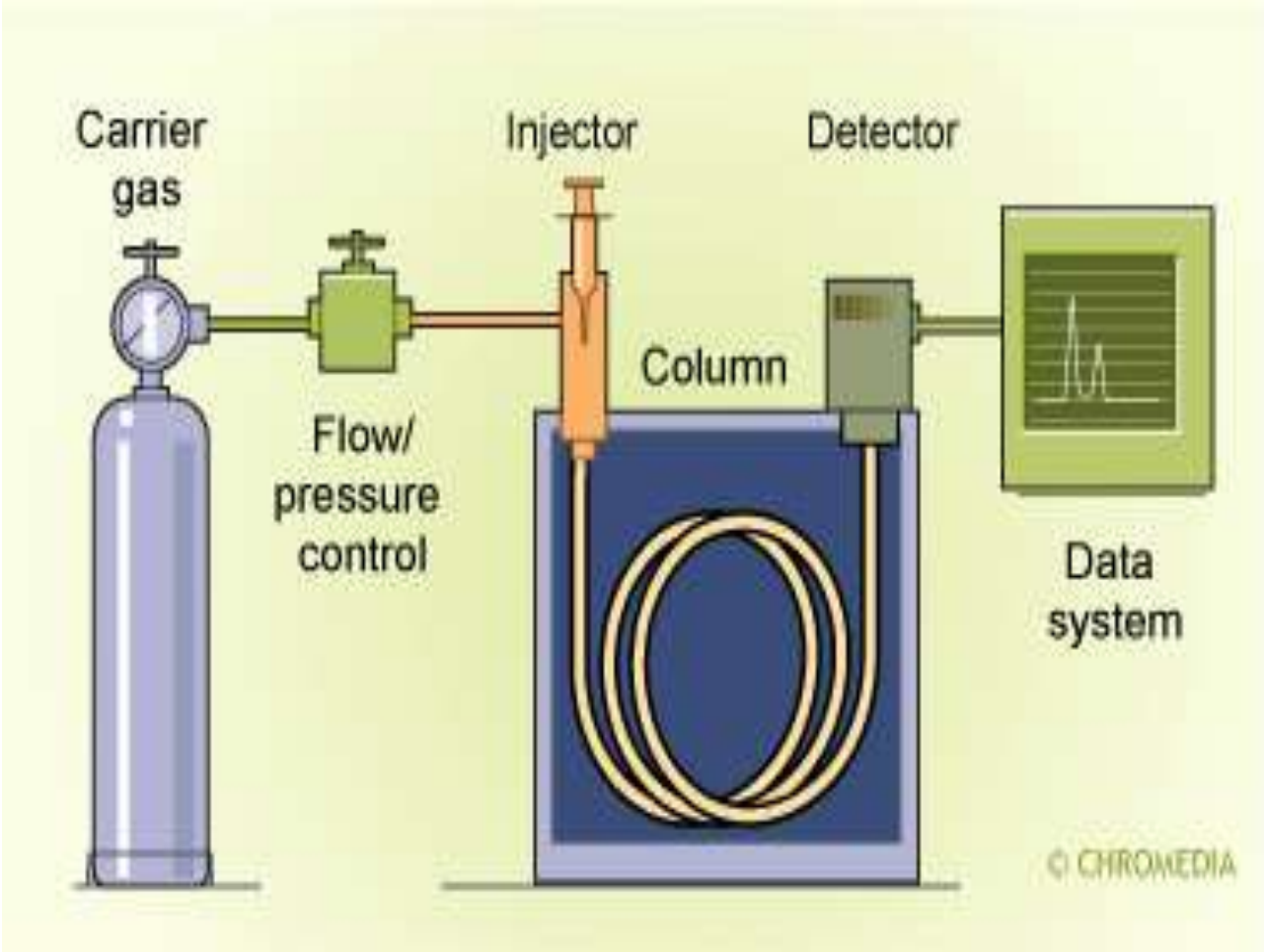
 The regulated carrier gas is supplied to the inlet and subsequently flows through the column and into the detector

 The sample is injected into the (usually) heated injection port where it is volatilized and carried into the column by the carrier gas

- The sample is separated inside the column - usually a long silica based column with small internal diameter. The sample separates by differential partition of the analytes between the mobile and stationary phases, based on relative vapor pressure and solubility in the immobilized liquid stationary phase

- On elution from the column, the carrier gas and analytes pass into a detector, which responds to some physicochemical property of the analyte and generates an electronic signal measuring the amount of analyte present

- **The data system then produces an integrated chromatogram**
- **Gas chromatography uses ovens that are temperature programmable. The temperature of the GC oven typically ranges from 5 ° C to 400 ° C but can go as low as -25 ° C with cryogenic cooling**



A gas chromatograph with a headspace sampler

Advantages

- Fast analysis
- High efficiency – leading to high resolution
- Sensitive detectors (ppb)
- Non-destructive – enabling coupling to Mass Spectrometers (MS) - an instrument that measures the masses of individual molecules that have been converted into ions, i.e. molecules that have been electrically charged
- High quantitative accuracy and requires small samples.

Disadvantages

- Limited to volatile samples
- Not suitable for samples that degrade at elevated temperatures (thermally labile)
- Not suited to preparative chromatography
- Requires MS detector for analyte structural elucidation (characterization)
- Most non-MS detectors are destructive

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

What is Liquid Chromatography?

- **Liquid chromatography is a separation technique that involves: the placement (injection) of a small volume of liquid sample into a tube packed with porous particles (stationary phase) where individual components of the sample are transported along the packed tube (column) by a liquid.**

- **The components of the sample are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.**

- **High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture.**

- **It relies on pumps to pass a pressurized liquid solvent (mobile phase) containing the sample mixture through a column filled with a solid adsorbent material (stationary phase).**

- **Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.**

- **The active component of the column, the adsorbent, is typically a granular material made of solid particles (e.g. silica, polymers, etc.), 2–50 micrometers in size.**

- **The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles.**

- **The pressurized liquid is typically a mixture of solvents (e.g. water, acetonitrile and/or methanol) and is referred to as a "mobile phase".**

- **Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent.**

- **These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination.**

- **HPLC instrument typically includes a sampler, pumps, and a detector.**

- **The sampler brings the sample mixture into the mobile phase stream which carries it into the column.**

- **The pumps deliver the desired flow and composition of the mobile phase through the column.**

- **The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components.**

- **A digital microprocessor and user software control the HPLC instrument and provide data analysis.**

- **Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or based on mass spectrometry.**

- **Most HPLC instruments also have a column oven that allows for adjusting the temperature at which the separation is performed.**

- Common mobile phases used include any miscible combination of water with various organic solvents (the most common are acetonitrile and methanol). Some HPLC techniques use water-free mobile phases.

- The aqueous component of the mobile phase may contain acids (such as formic, phosphoric or trifluoroacetic acid) or salts to assist in the separation of the sample components.

- The composition of the mobile phase may be kept constant ("isocratic elution mode") or varied ("gradient elution mode") during the chromatographic analysis.

- **Isocratic elution is typically effective in the separation of sample components that are not very different in their affinity for the stationary phase.**

- **In gradient elution the composition of the mobile phase is varied typically from low to high eluting strength.**

- **The eluting strength of the mobile phase is reflected by analyte retention times with high eluting strength producing fast elution (=short retention times).**

- **Many different types of columns are available, filled with adsorbents varying in particle size, and in the nature of their surface.**

- **The chosen composition of the mobile phase (also called eluent) depends on the intensity of interactions between various sample components ("analytes") and stationary phase.**

Components – HPLC

1. Pump

- The role of the pump is to force a liquid (called the mobile phase) through the liquid chromatograph at a specific flow rate, expressed in milliliters per min (mL/min).

- Normal flow rates in HPLC are in the 1- to 2-mL/min range. Typical pumps can reach pressures in the range of 6000-9000 psi (400- to 600-bar).

- During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient)

2. Injector

- The injector serves to introduce the liquid sample into the flow stream of the mobile phase.

- Typical sample volumes are 5- to 20-microliters (µL).

- The injector must also be able to withstand the high pressures of the liquid system.

- An autosampler is the automatic version for when the user has many samples to analyze or when manual injection is not practical.

3. Column

- the column's stationary phase separates the sample components of interest using various physical and chemical parameters.

- The small particles inside the column are what cause the high backpressure at normal flow rates.

- The pump must push hard to move the mobile phase through the column and this resistance causes a high pressure within the chromatograph.

4. Detector

- The detector can see (detect) the individual molecules that come out (elute) from the column.

- A detector serves to measure the amount of those molecules so that the chemist can quantitatively analyze the sample components.

- The detector provides an output to a recorder or computer that results in the liquid chromatogram (i.e., the graph of the detector response).

5. Computer

- Frequently called the data system, the computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative analysis).

- There are two variants in use in HPLC depending on the relative polarity of the solvent and the stationary phase.
  1. Normal phase HPLC
  2. Reversed phase HPLC

Normal phase HPLC

- The column is filled with tiny silica particles, and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm.

- **Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will.**

- **The non-polar ones will therefore pass more quickly through the column.**

- **Although it is described as "normal", it isn't the most commonly used form of HPLC.**

Reversed phase HPLC

- In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them.

- A polar solvent is used - for example, a mixture of water and an alcohol such as methanol.

- In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column.

- **There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution.**

- **Polar molecules in the mixture will therefore spend most of their time moving with the solvent.**

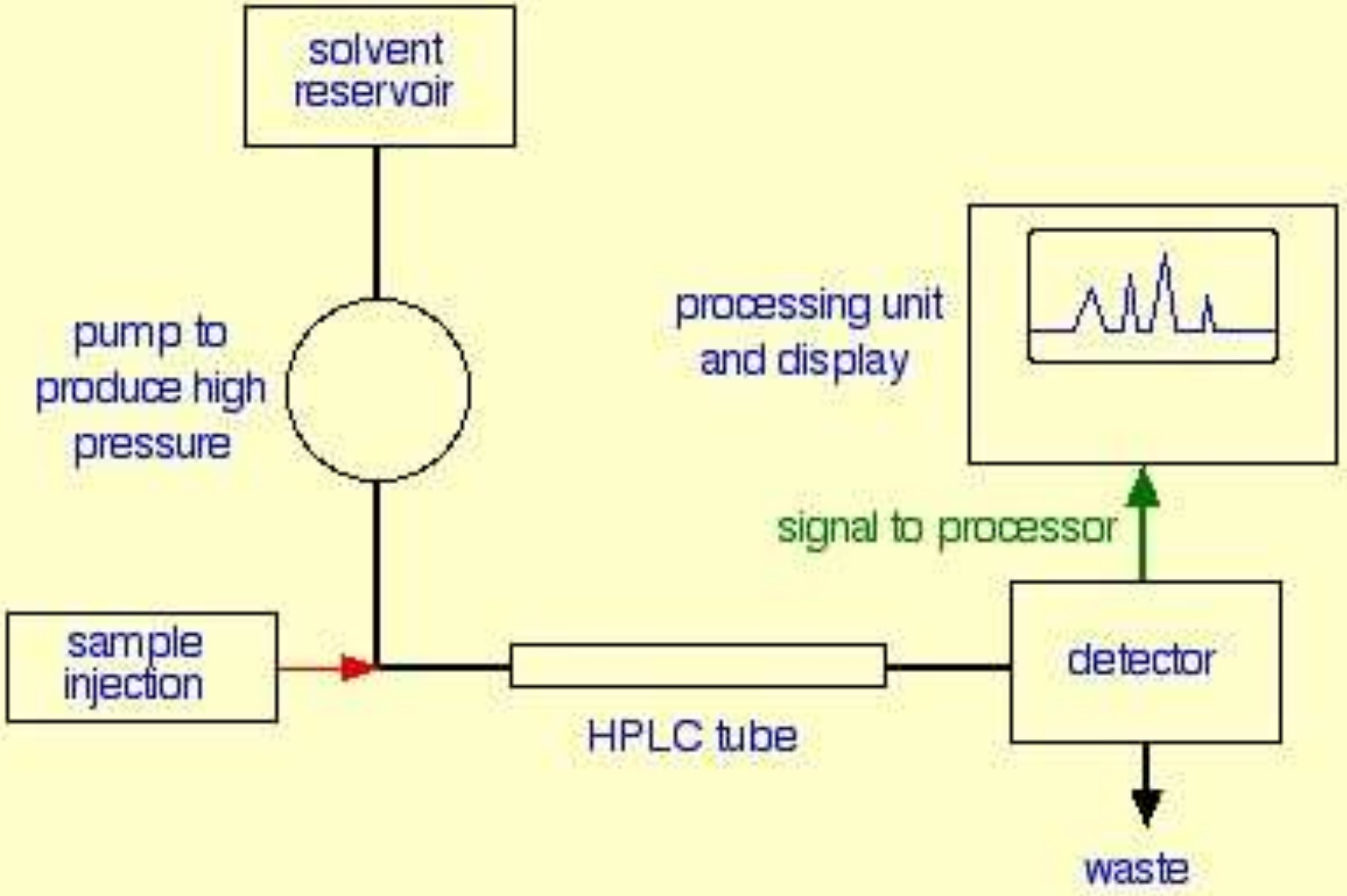
- **Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces.**

- **They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example.**

- **They therefore spend less time in solution in the solvent and this will slow them down on their way through the column. That means that now it is the polar molecules that will travel through the column more quickly.**

- **Reversed phase HPLC is the most commonly used form of HPLC.**

A flow scheme for HPLC



1. Injection of the sample

- **Injection of the sample is entirely automated,**

2. Retention time

- **The time taken for a particular compound to travel through the column to the detector is known as its *retention time*.**

- **This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.**

- **Different compounds have different retention times.**

- **For a particular compound, the retention time will vary depending on:**

  - **the pressure used (because that affects the flow rate of the solvent)**
  - **the nature of the stationary phase (not only what material it is made of, but also particle size)**
  - **the exact composition of the solvent**
  - **the temperature of the column**

- **That means that conditions have to be carefully controlled if you are using retention times as a way of identifying compounds.**

3. The detector

- There are several ways of detecting when a substance has passed through the column.

- A common method which is easy to explain uses ultra-violet absorption. Many organic compounds absorb UV light of various wavelengths and detected by using a UV detector.

- Detector can be connected to a mass spectrometer. There it will give a fragmentation pattern which can be compared against a computer database of known patterns. That means that the identity of a huge range of compounds can be found without having to know their retention times.

Types of Detector

1. **Refractve index (IR)&– Measures the change of refractive index of the eluent from the column with respect to pure mobile phase.**
2. **Ultraviolet (UV) – depends on absorption of UV ray energy by the sample.**
3. **Fluorescence - Fluorescence rays emitted by sample after absorbing incident light is measured. Xenon arc lamp is used to produce light for excitation. Only suitable for compounds which can produce florescence.**

4. **Photodiode Array (PDA) Detectors** – the range of detectors extends from UV, visible and to some extent to IR region. Higher sensitivity and measures the entire absorption range. It gives scan of entire spectrum.

5. **Electrochemical detectors** – Specially suitable to estimate oxidisable & reducible compounds. When compound is either oxidized or reduced, the chemical reaction produces electron flow, which is measured as current.

6. **Mass spectrometry**

4. Interpreting the output from the detector

- **The output will be recorded as a series of peaks - each one representing a compound in the mixture passing through the detector and absorbing UV light.**

- **HPLC is optimum for the separation of chemical and biological compounds that are non-volatile, Typical non-volatile compounds are:**
- **Pharmaceuticals like aspirin, ibuprofen, or acetaminophen (Tylenol)**
- **Salts like sodium chloride and potassium phosphate**
- **Proteins like egg white or blood protein**
- **Organic chemicals like polymers (e.g. polystyrene, polyethylene)**
- **Heavy hydrocarbons like asphalt or motor oil**
- **Many natural products such as ginseng, herbal medicines, plant extracts**
- **Thermally unstable compounds such as trinitrotoluene (TNT), enzymes**